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Ewing's sarcoma: General insights from a rare model

Ewing's sarcoma is characterized by the presence of fusion oncoproteins involving *EWSR1* and an *ETS* gene, most commonly *FLI1*. In this issue of *Cancer Cell*, Smith et al. have combined RNA interference with expression profiling to study the pattern of gene expression downstream of the most common of these fusions, EWS/FLI. Using this strategy, Smith et al. have identified a homeobox gene, *NKX2.2*, which is both highly expressed in Ewing's sarcoma and essential for the transforming activity of EWS/FLI.

Ewing's sarcoma (ES) is a rare cancer of children and young adults. It arises most frequently in bones, but it also can occur in a variety of soft tissue sites. It has a high propensity for metastasis, particularly to lung, bone, and bone marrow. While some patients can be cured with multimodality therapy, the substantial proportion of patients with metastatic disease usually present an insuperable clinical problem. Our understanding of ES biology is a tantalizing mix of knowledge and ignorance. For example, the cell of origin of ES is unknown as is the reason for its peculiar anatomic distribution. Although ES expresses some neural markers, there is no evidence that ES arises from a neural progenitor. In contrast, a great insight into ES biology came with the discovery that ES tumors typically express a chimeric transcription factor derived from *EWSR1* and *FLI1* (May et al., 1993; Delattre et al., 1992). This fusion forms as a result of the translocation between chromosomes 11 and 22 commonly observed in ES. *FLI1* is a member of the *ETS* family of transcription factors, and those ES tumors that lack the EWS/FLI fusion typically have a variant translocation involving one of several other *ETS* family members, most commonly *ERG*. *EWSR1* encodes a nuclear protein of unknown function. The fusion protein is comprised of the N-terminal portion of the EWSR1 protein linked to the DNA binding domain of FLI1. The chimeric protein has transforming activity in cell culture assays, but much remains to be learned about the biochemical properties of the fusion gene.

Despite the incomplete nature of our understanding of the biochemistry and cell biology of ES, it seems safe to conclude that the translocation that produces EWS/FLI is the key genetic event in the development of this cancer and that the presence of EWS/FLI transforms cells in large part through transcriptional dysregulation. From this perspective, ES biology resembles that of many leukemias and other sarcomas that arise as the consequence of chromosome translocations encoding chimeric transcription factors (Helman and Meltzer, 2003). Interestingly, both *FLI1* and *ERG* can act as leukemia oncogenes. The burning question in ES biology then becomes the elucidation of the mechanism by which EWS/FLI leads to malignant transformation. The answer, it seems, must lie in the pattern of gene expression driven by EWS/FLI and the specific pathways that are dysregulated as a result. Of course, the hope is that essential EWS/FLI target genes will be identified and that these may guide the development of new therapies for ES.

Determining genes associated with EWS/FLI-mediated malignant transformation

While this seems like a fairly straightforward research agenda, there is no way to introduce EWS/FLI into ES progenitor cells, since their nature is unknown. Even though many EWS/FLI targets have been found in heterologous models systems, studies in surrogates such as NIH 3T3 cells are not completely satisfactory, since the effects of EWS/FLI may well be context dependent, and heterologous

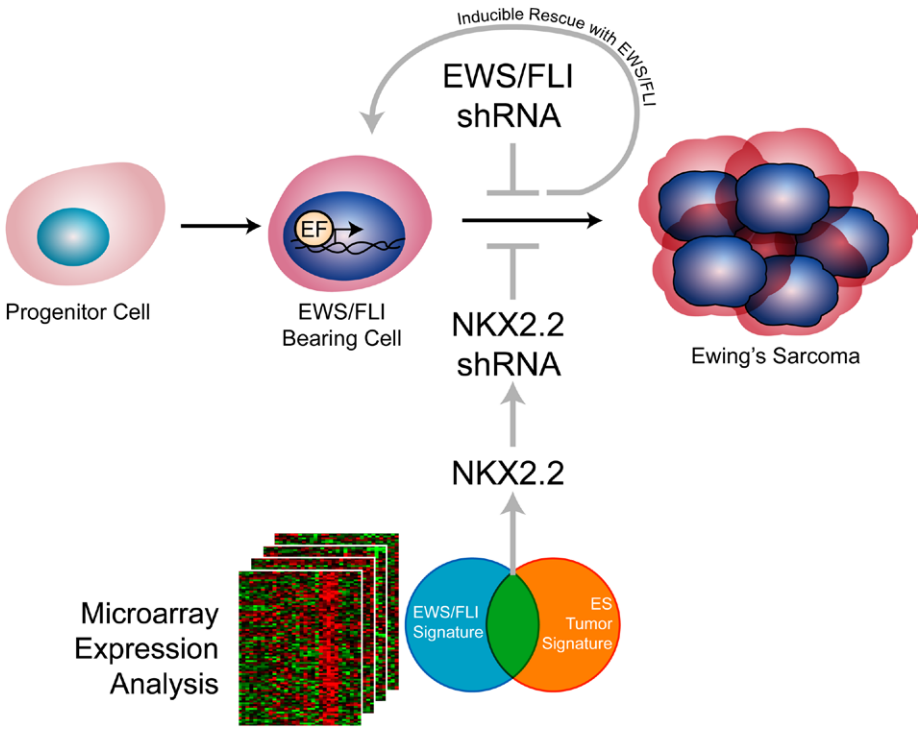

Figure 1. Ewing's sarcoma arises from an unknown progenitor cell that acquires the EWS/FLI translocation

The translocation-bearing cell ultimately gives rise to clinically evident Ewing's sarcoma as a consequence of the effects of EWS/FLI on gene expression and, most likely, the acquisition of additional genetic alterations. In order to identify a gene expression signature associated with EWS/FLI, the fusion gene was targeted with a stably expressed shRNA. This caused loss of transformation, which could be rescued by an inducible EWS/FLI expression construct. Microarray expression analysis was used to monitor the effect of these manipulations. The resulting EWS/FLI signature was compared with the expression signature of Ewing's sarcoma tumors. NKX2.2 was identified by this bioinformatics analysis and then targeted with a shRNA. This caused loss of the transformed phenotype in several ES cell lines, suggesting that its expression is driven (directly or indirectly) by EWS/FLI and that it is essential for the oncogenic properties of EWS/FLI. This strategy could in principle be applied to other similar fusion genes.

systems cannot be expected to reproduce the biology of ES (Deneen et al., 2003). This is a general problem that plagues investigators studying the biology of fusion proteins in other systems where it may be difficult or impossible to obtain the appropriate precursor cell population for gene transfer studies. The advent of RNA interference has opened up a new realm of possibilities. Knocking down EWS/FLI expression in ES cells becomes a very attractive option, and it is this approach that has been taken by Smith et al. (2006). They identified an ES cell line, A673, that tolerates silencing EWS/FLI by a shRNA construct targeted to the 3′ UTR of *FLI1* (*FLI1* itself is not expressed in these cells). A673 cells that express the EWS/FLI shRNA construct still can be cultured, but they lose their transformed phenotype, as evidenced by diminished clonogenicity in soft agar and loss of xenograft tumorigenicity in mice. These phenotypic effects were reversed by rescue with an inducible EWS/FLI construct. The availability of this "inducible rescue" system allowed Smith et al. to use expression microarrays to examine the effects of silencing and then restoring EWS/FLI expression (Figure 1). The result is a list of genes (both downregulated and upregulated by EWS/FLI) that, at least in this cell line, can be placed downstream of EWS/FLI. Although it is impossible for Smith et al. to say which of these are direct targets of EWS/FLI, the EWS/FLI expression signature does show significant overlap with that of ES tumors by bioinformatics analysis (Khan et al., 2001). Moreover, even simple inspection of their gene list reveals genes that are well known to be expressed in ES such, as the neuropeptide Y receptor. These results are also consistent with the observations of others (Hu-Lieskovan et al., 2005) who found that expression of EWS/FLI in a rhabdomyosarcoma cell line induced neural markers. Taken together, these findings suggest that the neural phenotype of ES may be a consequence of EWS/FLI activity rather than a direct clue to the identity of the ES progenitor cell.

Importance of NKX2.2 in EWS/FLI-mediated tumorigenicity

If the transforming effects of EWS/FLI are a consequence of dysregulated gene expression, then the list of genes that constitute the EWS/FLI signature should contain important mediators of this process. Smith et al. tested one of these candidates, the homeobox gene *NKX2.2*, in some detail. They demonstrated that silencing *NKX2.2* in A673 cells causes loss of clonogenicity in soft agar and loss of tumorigenicity in mice. Moreover, the same effect is observed in other ES cell lines, suggesting that *NKX2.2* may indeed be of general importance as a mediator of EWS/FLI transformation. Interestingly, published ES tumor expression profiles demonstrate frequent expression of *NKX2.2* (Baird et al., 2005). However, forced expression of *NKX2.2* is not sufficient to rescue the effects of EWS/FLI silencing in A673 cells, leading Smith et al. to conclude that *NKX2.2* is necessary but not sufficient for the growth of ES. Although homeobox genes are known oncogenes in certain cancers, *NKX2.2* has never been associated with tumorigenesis. This observation opens up the possibility of reconstructing the regulatory cascade downstream of EWS/FLI. If *NKX2.2* is indeed a mediator of EWS/FLI transformation, then it can be predicted that some portion of the EWS/FLI gene signature should consist of genes that are direct targets of *NKX2.2*.

Broader applications of the "inducible rescue" approach

Although ES is a rare tumor, the importance of this study extends beyond its contribution to our understanding of ES itself. First, it should be noted that the "inducible rescue" approach taken by Smith et al. can, in principle, be generalized to any of the myriad of oncogenes that have been identified in any cancer and may prove to be particularly well suited to the many oncogenes that act as transcription factors. By combining two of the most powerful new technologies in cancer research, RNAi and microarray analysis, it should prove possible to dissect the regulatory networks downstream of any oncogene and to do this in a reasonably authentic cellular context. Second, insights into the transforming actions of *ETS* family fusion genes are of broad relevance. Although the role of *ETS* genes in leukemogenesis has long been known, very recently they have been linked to prostate cancer by the discovery of *ETS* gene fusions in that disease (Tomlins, et al., 2005), raising the possibility that such gene fusions are more important in the common solid tumors than previously thought. Indeed, *ETS* gene fusions in prostate cancer now appear to be the most common oncogenic translocation known. In this context, the oncogenic mechanisms of *ETS* gene fusions takes on a new and broader importance. The results of Smith et al. demonstrate a general approach to systematically define those mechanisms and identify pathways that can be therapeutically targeted in cancers that are driven by oncogenic transcription factors.

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